Chapter 5 **Inflammatory Angiopoietin Response in Deceased Brain Dead Donors**

Lyan G. Koudstaal, Willemijn N. Nijboer, Johannes G. Burgerhof, Jaap J. Homan van der Heide, Vincent B. Nieuwenhuijs, Maarten W. Nijsten,Harry van Goor, Rutger J. Ploeg, Henri G.D. Leuvenink

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Abstract

Background: Kidneys retrieved from deceased brain dead (DBD) donors show inferior function compared to living donors. Bacterial translocation and endotoxemia as well as inflammation are frequent in DBD donors. Recently it was shown that in endotoxic patients the angiopoietin levels are influenced. We hypothesized that the anti-inflammatory protein angiopoietin-1 (ang-1) and the pro-inflammatory angiopoietin 2 (ang-2) are shifted towards inflammation in DBD donors compared to living donors. Therefore, we studied serum vascular endothelial growth factor (VEGF), ang-1 and ang-2 in DBD donors using living kidney donors as controls. Lipopolysaccharide Binding Protein (LBP) was used to quantify endotoxemia.

Methods: Serum was collected just after confirmation of brain death diagnosis (T0) and immediately prior to organ recovery (T1) from 30 consecutive DBD donors. Twenty living donors were asked informed consent for two blood samples, obtained at the beginning of the operation and immediately prior to retrieval of the donor kidney.

Results: DBD donors had higher median serum LBP, VEGF and ang-2 levels compared to living donors. Higher ang-1 levels were observed just after brain death diagnosis compared to living donors. Serum levels of LBP and ang-2 were correlated with a Spearman's ρ of 0.6. Importantly, serum ang-2 levels in the DBD donor predicted the chance on rejection in the first year after kidney transplantation with an odds ratio at T0 1.38 and at T1 1.50 ($P<0.05$).

Conclusions: The angiopoietin balance in brain dead donors is modulated progressively towards inflammation during the period of brain death prior to organ recovery.

Introduction

Organs recovered from heart beating deceased brain dead (DBD) donors show inferior organ function and a higher rate of acute rejection compared to those obtained from living donors (1-4). Both in humans and in experimental models, brain death induces a progressive inflammatory response in potential donor organs such as kidney, liver and intestine (5-7). This response coincides with systemic higher levels of circulating cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor alpha, vascular endothelial growth factor, and macrophage chemoattractant protein-1 (8-10). The exact mechanism responsible for these pro-inflammatory changes and their detrimental effect on transplant outcome are yet unknown.

The intestine is considered to be a crucial player in the development of distant organ injury. In several conditions, such as severe burns, brain injury, acute pancreatitis and Acute Respiratory Distress Syndrome (ARDS), enhanced intestinal permeability is associated with distant organ injury (11-15). Several human studies have provided evidence that DBD donors have a higher endotoxin load. Bacterial translocation and elevated endotoxin levels are also frequent in DBD donors (16). Almost half of the donors have positive cultures of the ileocoecal lymph node (17).

Recently, a link between endotoxemia and angiopoietin 1 (ang-1) and angiopoietin 2 (ang-2) has been established. In humans, LPS is a triggering factor for ang-2 release (18). Ang-1 and ang-2 both are regulatory proteins which play an important role in vascular inflammation. The angiopoietin–Tie ligand-receptor system is crucial in regulating vascular integrity and quiescence (19). Ang-1 dampens the inflammatory response while ang-2 boosts it (20). Ang-1 has the ability to seal the vasculature, act as an anti-inflammatory agent, protect against cardiac allograft arteriosclerosis and renal fibrosis, and promote wound healing (21-24). Ang-2 is associated with an increased morbidity and mortality during sepsis. A model has been reported which indicates that a balanced ang-1/ang-2 ratio determines the functional status of the vasculature (25). It has also been shown that in vivo endotoxemia triggers functional inhibition of the angiopoietin pathway by reducing ang-1 expression and inducing ang-2 levels and that this response may contribute to enhanced vascular leakage during sepsis (26).

We hypothesized that in heart beating brain dead donors the inflammatory proteins ang-1 and ang-2 are shifted towards inflammation. Living kidney donors were used for comparison as controls. Further, we questioned whether the ang-1 and ang-2 status in the DBD donor can be used as a prognostic tool to predict renal function after transplantation including delayed graft function (DGF) and rejection. Therefore, we studied serum ang-1 and ang-2 in 30 DBD donors and 20 living kidney donors and linked the results to clinical kidney transplant outcome. We studied serum lipopolysaccharide Binding Protein (LBP) as a quantification of endotoxemia in kidney donors serum and vascular endothelial growth factor

(VEGF), because it has been shown ang-2 primes the endothelium to respond to VEGF (27-33).

Methods

PATIENTS AND SERUM SAMPLES

Serum samples were prospectively, consecutively collected during organ procurement procedures from a series of heart beating DBD donors, from 2004 through 2007. Selection criteria were donor age <65y and a cold ischemia time <30h. All donors were declared brain dead on the intensive care. All samples were collected after declaration of brain death, according to the Dutch Transplantation Law. Baseline samples were collected just after brain death diagnosis (T0). A second sample (T1) was obtained prior to organ recovery just before wash-out and preservation. Due to logistic reasons, at T0 in four DBD donors no serum could be obtained. Twenty living kidney donors were asked informed consent for two blood samples: a baseline sample (T0) just before the donor operation (T0) and a second sample (T1) at the time of kidney recovery during the donor operation just prior to retrieval. All samples were kept on ice, centrifuged for 20 minutes at 1500 x g and stored at -80˚C until analysis. Clinical donor variables were recorded and one year-follow up of kidney function after transplantation was received from recipient hospitals. As the end points for clinical outcome were used: Delayed graft function (DGF), defined as the need for dialysis in the first week after transplantation; rejection, defined as biopsy proven rejection in the first year and serum creatinine in the recipient on day 14 after transplantation. All grades of interstitial and vascular rejection were included. Borderline rejection was excluded. Biopsies were scored during routine clinical practice by a blinded pathologist according to the BANFF classification.

SERUM LIPOPOLYSACCHARIDE BINDING PROTEIN (LBP)

To evaluate LBP in serum samples from all patients included in the study an enzyme-linked immunosorbent assay (ELISA) test kit (HyCult Biotechnology, Uden, The Netherlands) for Human Lipopolysaccharide Binding Protein (LBP) was used, according to the manufacturer's instructions, with an upper reference value for healthy individuals of 10 µg/ml. All samples were tested in duplicate and read at 450 nm, in a microplate reader (Victor3, 1420 multilabel counter, Perkin Elmer).

SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

To determine VEGF levels, serum samples were measured using a multiplex bead sandwich immunoassay (Biosource, Invitrogen, Carlsbad, CA) which was analyzed using a Luminex 100 instrument (Luminex, Austin, TX).

SERUM ANGIOPOIETIN 1 AND 2

Human ang-1 and ang-2 enzyme-linked immunoassay (ELISA) test kits (R&D systems, Minneapolis, USA) were used according to the manufacturer's instructions, to evaluate ang-1 and ang-2 in serum samples from all patients included in this study. The upper reference value in healthy individuals for ang-1 is

6 ng/ml and ang-2 is 2.5 ng/ml (34). All samples were tested in duplicate and read at 450 nm using a micro-plate reader (Victor3, 1420 multi-label counter, Perkin Elmer).

STATISTICAL METHODS

Statistical analysis was performed using the computer program SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as median and 25 and 75 percentiles. Statistical comparisons between groups were performed using a T-test if data had a normal distribution; otherwise groups were compared using the Mann-Whitney U test. Dichotomous variables were compared using Fisher's test. The association between LBP, VEGF, ang-1 and ang-2 was tested with Spearman's rho. For linear regression a normal distribution of the residues was checked using a normal probability plot. Multivariate stepwise logistic regression with the log likelihood test was used for the dichotomous variables, DGF and rejection. For serum creatinine in the recipient on day 14 after transplantation multivariate linear regression was used. In all models as covariates As covariates donor age, HLA-mismatches, sex, cold ischemia time were tested. All differences were considered to be significant at $P<0.05$.

Results

In this study 30 DBD donors and 20 living renal donors were included; all kidneys obtained from these donors were transplanted. The median time between T0, declaration of brain death and T1, at the time of kidney recovery was 11 hours (10-13). The cause of death was a cerebrovascular accident (CVA) in 19 donors and 11 donors with a trauma capitis or other cause. The occurrence of DGF and serum creatinine after 14 days was recorded for all patients; however occurrence of rejection in the first year was analyzed in 48 patients. Ten DBD donors experienced DGF compared to one in the living donors and six DBD donors experienced rejection compared to three living donors ($P<0.05$). One patient experienced graft loss four weeks post-transplant due to mycotic aneurysm, two patients died with a functioning graft and two patients were referred to non-participating hospitals. Donor characteristics are shown in table 1.

In the serum of DBD donors we found significantly higher LBP, VEGF, ang-1 and ang-2 levels at T0 ($P<0.001$) and higher LBP, VEGF and ang-2 levels at T1 ($P<0.001$). Interestingly, the ang-1 levels decreased significantly during the brain death period ($P<0.001$). In DBD donors at T1 the ang-1/ang-2 ratio was decreased compared to living donors ($P<0.001$). In DBD donors the ang-1/ang-2 ratio decreased from T0 to T1 (Figure 1) ($P<0.001$).

Table 1 Characteristics of DBD and Living Donors and Kidney Recipients

	DBD Donors	Living donors	P-value
Gender (M:F)[c]	10:20	7:13	0.903
Age (years) [b]	49 (42-57)	50 (44-58)	0.246
Cause of death			
CVA	19		
Trauma/Other	11		
Serum creatinine T0 (µmol/L)[a]	60 (49-87)	64 (60-70)	0.782
Serum creatinine T1 (µmol/L)[a]	57 (48-72)	64 55-68)	0.357
Post-transplant Parameters			
Cold Ischemia Time (hours)[a]	198 (15-23)	3 (2-3)	0.001
HLA mismatch[a]	2 (2-3)	3 (2-4)	0.01
First transplant[a]	28/30	20/20	0.0001
Serum Creatinine day 14 (µmol/L)[a]	166 (114-556)	133 (116-185)	0.111
Delayed Graft Functionc	10	1	0.033
Rejection[c]	6	3	0.716
Immunosuppressive Treatment			
Prednisolon/Mycophenolate mofetil/Cyclosporine	24	20	
Prednisolon/Mycophenolate mofetil/ Tacrolimus	2	0	
Prednisolon/Cyclosporine/ FTY720	1	0	
Prednisolon/ Mycophenolate mofetil/ Rapamycin	1	0	
Antithymocyte globulin	2	2	
Daclizumab	4	9	
Basiliximab	3	2	
Unknown	2	0	

Mann Whitney U test [a] ; Student's T-test [b]; Fisher's test [c] ;Results of age and serum creatinine are expressed as median and 25 and 75 percentiles.

In DBD donors we observed high ang-1 levels at T0 in all donors who died from a stroke (n=19). At T0 median ang-1 levels were 24 (17-31) ng/ml in donors who died from a CVA compared to 9 (6-14) ng/ml in donors with another cause of death ($P<0.05$). These medians did not differ anymore during organ recovery. In donors respectively a CVA or with another cause of death, ang-1 levels were at T1 6(3-8) ng/ml vs. 8 (5-10) ng/ml (ns).

We also studied the association between ang-1 and LBP and the association between ang-2 and LBP. We found an association at T0 of LBP with ang-2 with a Spearmans ρ of 0.622 ($P<0.001$) and at T1 of 0.603 ($P<0.001$) (Figure 2).

To evaluate whether levels of ang-1, ang-2 or the ang-1/ang-2 ratio in the DBD donor group had an independent effect on outcome after transplantation linear and logistic stepwise multivariate regression models were built. DGF, rejection and serum creatinine on day 14 following transplantation were studied. As covariates donor age, HLA-mismatches, sex, cold ischemia time were tested. Only donor age did predict DGF, with an OR of 1.1 ($P<0.05$). Elevated ang-2 levels were

associated with an increased risk of rejection with an odds ratio at T0 1.377 and at T1 1.499 (P<0.05). In contrast, donor serum creatinine did not predict kidney outcome after transplantation. DGF and serum creatinine on day 14 were not predicted by LBP, VEGF, ang-1, ang-2, or the ang-1/ang-2 ratio. ROC analyses are presented in Figure 3 to evaluate the prognostic effect of serum ang-2 on rejection in the first year after transplantation. The area under the curve for both time point was 0.8 (P<0.05). With a angiopoietin value of 4.5 ng/ml, the sensitivity is 0.8 with a false negative rate of 0.2.

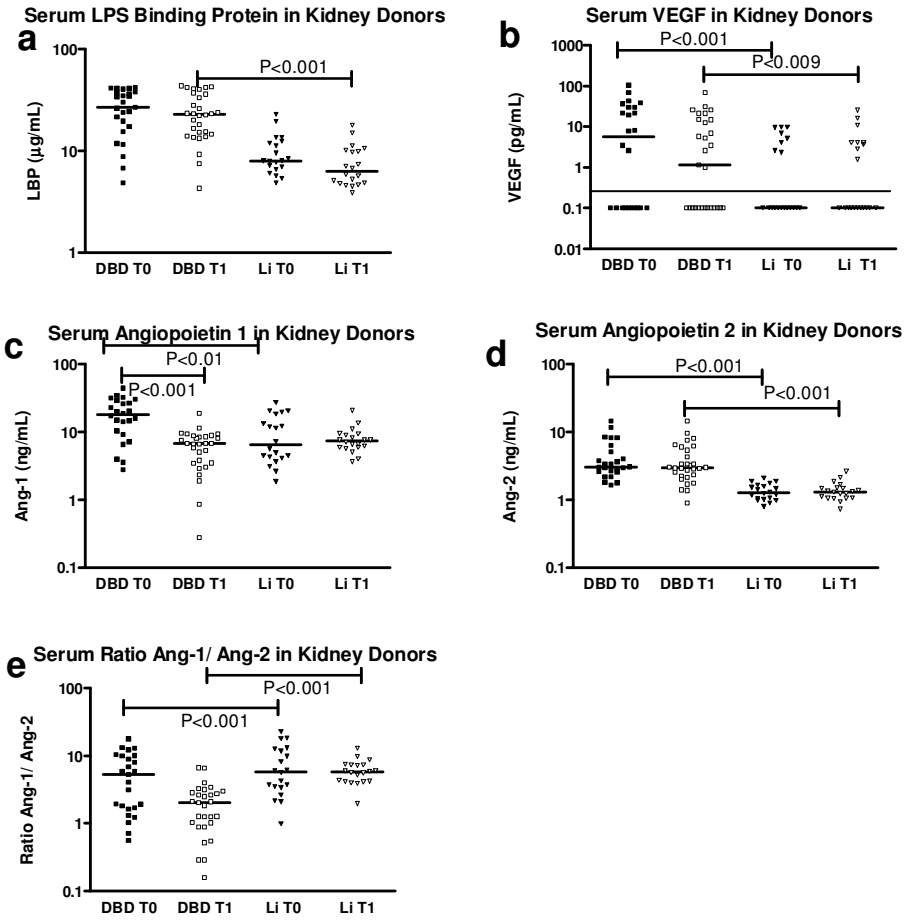


Figure 1 Dot plots of Lipopolysaccharide Binding Protein a, Vascular Endothelial Growth Factor b, angiopoietin-1 c, angiopoietin-2 d and ratio ang1/ang2 the median is shown. Baseline samples were collected just after brain death diagnosis (T0). A second sample (T1) was obtained during organ recovery just prior to wash-out and preservation.

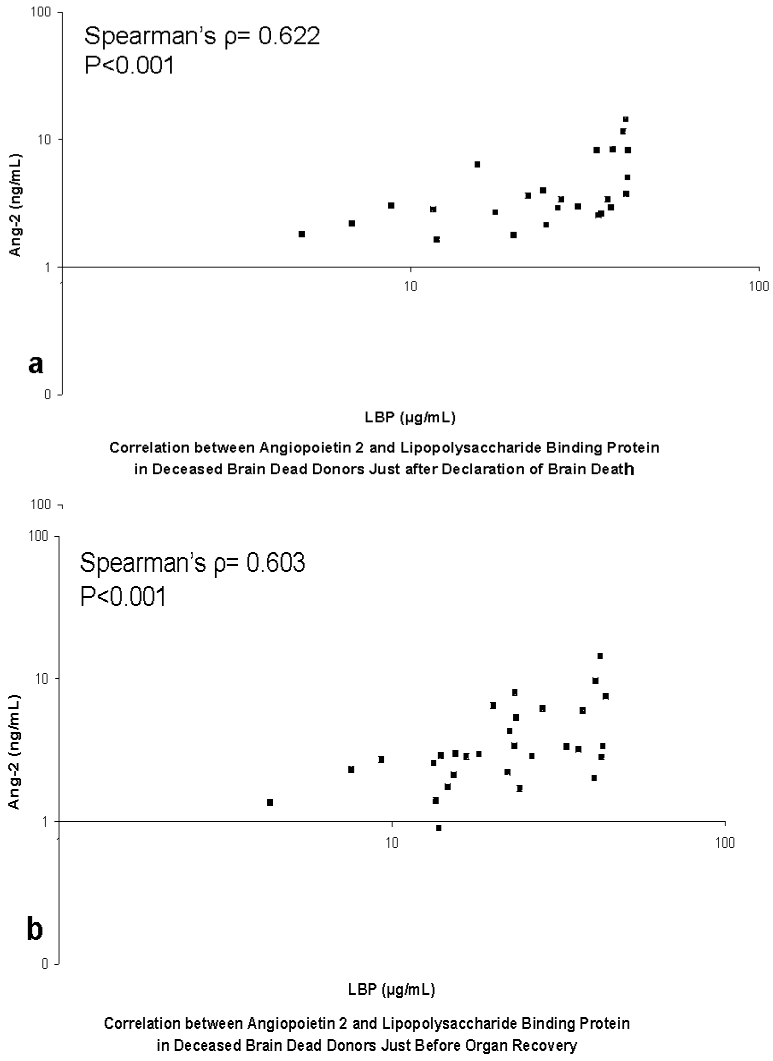
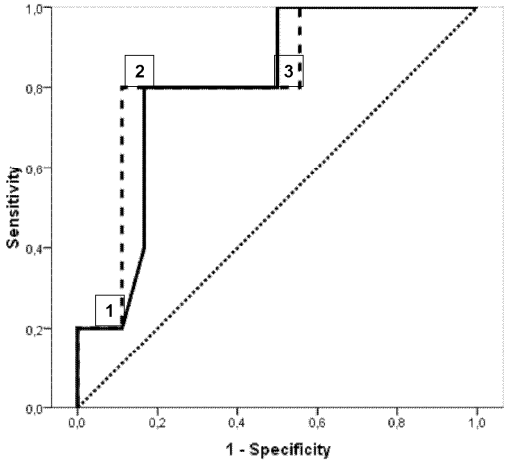


Figure 2 Dot plots correlation between Serum Lipopolysaccharide Binding Protein and Serum angiopoietin 2 in deceased brain dead donors (DBD) at T0, baseline just after brain death diagnosis a and T1 during organ recovery just prior to wash-out and preservation b.



Cut Point	Ang-2 (ng/ml)	True Positives	Fals Positives
1	8,4	0,2	0,1
2	4,5	0,8	0,2
3	2,9	1	0,50

ROC Curve Analyses of Angiopoietin 2 Predicting Rejection in the First Year after Transplantation

Figure 3 ROC analyses of angiopoietin-2 to predict rejection

Discussion

The cardinal finding of this study is the modulated angiopoietin balance in brain dead donors towards pro-inflammatory activation. Just after the diagnosis of brain death and prior to organ recovery, we found higher serum ang-2 levels compared with living donors. Interestingly, we also observed higher ang-1 levels just after brain death diagnosis compared to living donors. These ang-1 levels decreased during the period of brain death until the moment of organ recovery to levels comparable with living donors. Similarly, during the brain dead period the ratio of ang-1/ang-2 decreased significantly, shifting to pro-inflammatory activation. In addition, serum ang-2 levels in the DBD donor predicted the chance of rejection in the recipient. The area under the curve of the ROC analysis, was 0.8, showing a good discriminating potential of ang-2. Therefore, among the other prognostic factors, ang-2 can add relevant information for clinical decision-making to determine whether a kidney will be suitable for transplantation. Based only on this study, it is too premature to draw definite conclusion. Further research is needed to evaluate ang-2 prognostic value.

In this study we observed an enhanced endotoxin load as evidenced by the elevated serum LBP levels in DBD organ donors in contrast with living organ donors. This is in accordance with the observation that bacterial translocation is common in organ donors (35;36). LBP was shown to be a sensitive marker of prolonged endotoxin exposure (37), and our analysis revealed that prolonged exposure to endotoxins was present in the majority of heart beating DBD donors.

Serum ang-2 and VEGF are markedly elevated in several conditions such as sepsis and trauma (38;39). Angiopoietins play also a role in various kidney

diseases (40). In trauma patients with severe injury and systemic hypoperfusion, ang-2 is released within 30 minutes after trauma and high levels of ang-2 were associated with an activated endothelium, coagulation abnormalities, complement activation, and worse clinical outcome (41). In addition, in patients with acute lung injury (ALI) and ARDS high levels of circulating ang-2 are associated with pulmonary permeability oedema, occurrence and the severity of ALI/ARDS as well as with mortality (42;43). Furthermore, kidney grafts from living unrelated donors have high survival rates, despite a higher degree of HLA mismatching than is found in deceased grafts (44). The inferior survival of deceased donation cannot be attributed to differences in immunogenicity alone. Even when corrected for donor variables such as donor age, gender, race, terminal serum creatinine, shorter cold-ischemia times, cerebrovascular accident as the cause of death, and history of hypertension organs recovered from deceased donors show inferior outcome (45;46). The observed systemic inflammatory state and extend of injury in DBD donors is thought to induce worse outcome (47-51). The modulated inflammatory Angiopoietin response is in line with these observations.

In DBD donors the ang-1/ang-2 ratio is decreased and progressively shifted towards pro-inflammatory activation. Intervening in the ang-1/ang-2 balance may therefore be of therapeutical value to improve outcome after transplantation when kidneys from heart beating DBD donors are used. ang-2 neutralizing reagents have been developed as anti-angiogenic tumor drugs (52) and could also be used to decrease the pro-inflammatory status of the donor. Increasing the ang-1 concentration to restore the balance has a potent anti-inflammatory potential in animal models, although ang-1 therapies have also shown side effects like pulmonary hypertension (53;54).

Interestingly, hypertensive patients have higher levels of ang-1, even without target organ damage (55). In DBD donors we observed high ang-1 levels at T0 in all donors who died from a stroke (n=19). The elevated levels of ang-1 in donors who died from a stroke direct after declaration of brain dead, but not just before organ recovery, may reflects a protective response or reflects chronic vascular dysfunction in stroke patients.

We also found a strong association between LBP and ang-2 levels at both time points. The higher ang-2 levels, combined with the lower ang-1 levels just before organ recovery, are in accordance with triggering of ang-2 by administration of LPS to healthy volunteers and with the observation in mice, that endotoxemia triggers functional inhibition of the ang-1 pathway in vivo by reducing ang-1 expression and inducing ang-2 (56;57).

We conclude that the angiopoietin balance in brain dead donors is modulated progressively towards pro-inflammatory activation. Angiopoietin-2 could be a valuable marker to predict the quality of the renal graft as early as in the donor.

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